

CHAPTER II

JC20 Rec'd PCT/PTO SEP 21 2001

Preliminary Classification:

Proposed Class:

Subclass:

NOTE: "All applicants are requested to include a preliminary classification on newly filed patent applications. The preliminary classification, preferably class and subclass designations, should be identified in the upper right-hand corner of the letter of transmittal accompanying the application papers, for example 'Proposed Class 2, subclass 129.'" M.P.E.P., § 601, 7th ed.

**TRANSMITTAL LETTER
TO THE UNITED STATES ELECTED OFFICE (EO/US)
(ENTRY INTO U.S. NATIONAL PHASE UNDER CHAPTER II)**

PCT/US00/13292

12 May 2000

14 May 1999

INTERNATIONAL APPLICATION NO.

INTERNATIONAL FILING DATE

PRIORITY DATE CLAIMED

Surface Display of Selenocysteine-Containing Peptides

TITLE OF INVENTION

New England Biolabs, Inc., Karen E. Sandman and Christopher J. Noren

APPLICANT(S)

Box PCT

Assistant Commissioner for Patents

Washington D.C. 20231

ATTENTION: EO/US

CERTIFICATION UNDER 37 C.F.R. §§ 1.8(a) and 1.10*

(When using Express Mail, the Express Mail label number is mandatory;
Express Mail certification is optional.)

I hereby certify that, on the date shown below, this correspondence is being:

MAILING

- ☒ deposited with the United States Postal Service in an envelope addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231

37 C.F.R. § 1.8(a)

37 C.F.R. § 1.10 *

- ☐ with sufficient postage as first class mail.

- ☒ as "Express Mail Post Office to Addressee"

Mailing Label No. EL010481505US (mandatory)**TRANSMISSION**

- ☐ facsimile transmitted to the Patent and Trademark Office, (703)

Date: 9/21/01

Signature

Melissa A. Jackson

(type or print name of person certifying)

* Only the date of filing (§ 1.6) will be the date used in a patent term adjustment calculation, although the date on any certificate of mailing or transmission under § 1.8 continues to be taken into account in determining timeliness. See § 1.703(f). Consider "Express Mail Post Office to Addressee" (§ 1.10) or facsimile transmission (§ 1.6(d)) for the reply to be accorded the earliest possible filing date for patent term adjustment calculations.

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NOTE: To avoid abandonment of the application, the applicant shall furnish to the USPTO, not later than 20 months from the priority date: (1) a copy of the international application, unless it has been previously communicated by the International Bureau or unless it was originally filed in the USPTO; and (2) the basic national fee (see 37 C.F.R. § 1.492(a)). The 30-month time limit may not be extended. 37 C.F.R. § 1.495.

WARNING: Where the items are those which can be submitted to complete the entry of the international application into the national phase are subsequent to 30 months from the priority date the application is still considered to be in the international state and if mailing procedures are utilized to obtain a date the express mail procedure of 37 C.F.R. § 1.10 must be used (since international application papers are not covered by an ordinary certificate of mailing—See 37 C.F.R. § 1.8.

NOTE: Documents and fees must be clearly identified as a submission to enter the national state under 35 U.S.C. § 371 otherwise the submission will be considered as being made under 35 U.S.C. § 111. 37 C.F.R. § 1.494(f).

I. Applicant herewith submits to the United States Elected Office (EO/US) the following items under 35 U.S.C. § 371:

- a. ☒ This express request to immediately begin national examination procedures (35 U.S.C. § 371(f)).
- b. ☒ The U.S. National Fee (35 U.S.C. § 371(c)(1)) and other fees (37 C.F.R. § 1.492) as indicated below:

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2. Fees

| CLAIMS FEE | (1) FOR | (2) NUMBER FILED | (3) NUMBER EXTRA | (4) RATE | (5) CALCULATIONS |
|----------------------------|--|------------------|------------------|--------------------|------------------|
| <input type="checkbox"/> * | TOTAL CLAIMS | 20 - 20 = | 0 | $\times \$18.00 =$ | \$ 0.00 |
| | INDEPENDENT CLAIMS | 10 - 3 = | 7 | $\times \$80.00 =$ | 560.00 |
| | MULTIPLE DEPENDENT CLAIM(S) (if applicable) + \$270.00 | | | | 0.00 |
| BASIC FEE** | <input checked="" type="checkbox"/> U.S. PTO WAS INTERNATIONAL PRELIMINARY EXAMINATION AUTHORITY Where an international preliminary examination fee as set forth in § 1.482 has been paid on the international application to the U.S. PTO: <input type="checkbox"/> and the international preliminary examination report states that the criteria of novelty, inventive step (non-obviousness) and industrial activity, as defined in PCT Article 33(1) to (4) have been satisfied for all the claims presented in the application entering the national stage (37 C.F.R. § 1.492(a)(4)) \$100.00 <input checked="" type="checkbox"/> and the above requirements are not met (37 C.F.R. § 1.492(a)(1)) \$690.00 <input type="checkbox"/> U.S. PTO WAS NOT INTERNATIONAL PRELIMINARY EXAMINATION AUTHORITY Where no international preliminary examination fee as set forth in § 1.482 has been paid to the U.S. PTO, and payment of an international search fee as set forth in § 1.445(a)(2) to the U.S. PTO: <input type="checkbox"/> has been paid (37 C.F.R. § 1.492(a)(2)) \$710.00 <input type="checkbox"/> has not been paid (37 C.F.R. § 1.492(a)(3)) \$1000.00 <input type="checkbox"/> where a search report on the international application has been prepared by the European Patent Office or the Japanese Patent Office (37 C.F.R. § 1.492(a)(5)) \$860.00 | | | | 690.00 |
| | Total of above Calculations | | | | = 1250.00 |
| SMALL ENTITY | Reduction by 1/2 for filing by small entity, if applicable. Assertion must be made. (note 37 C.F.R. § 1.27) | | | | 625.00 |
| | Subtotal | | | | 625.00 |
| | Total National Fee | | | | \$ 625.00 |
| | Fee for recording the enclosed assignment document \$40.00 (37 C.F.R. § 1.21(h)). (See Item 13 below). See attached "ASSIGNMENT COVER SHEET". | | | | 40.00 |
| TOTAL | Total Fees enclosed | | | | \$665.00 |

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The PTO did not receive the following listed item(s) \$ 625.00
 Check Amount Received

*See attached Preliminary Amendment Reducing the Number of Claims.

- ☒ Attached is a ☒ check ☐ money order in the amount of \$ 625.00
☐ Authorization is hereby made to charge the amount of \$ _____
☐ to Deposit Account No. _____
☐ to Credit card as shown on the attached credit card information authorization form PTO-2038.

WARNING: Credit card information should **not** be included on this form as it may become public.

- ☐ Charge any additional fees required by this paper or credit any overpayment in the manner authorized above.

A duplicate of this paper is attached.

****WARNING:** "To avoid abandonment of the application the applicant shall furnish to the United States Patent and Trademark Office not later than the expiration of 30 months from the priority date: * * * (2) the basic national fee (see § 1.492(a)). The 30-month time limit may not be extended." 37 C.F.R. § 1.495(b).

WARNING: If the translation of the international application and/or the oath or declaration have not been submitted by the applicant within thirty (30) months from the priority date, such requirements may be met within a time period set by the Office. 37 C.F.R. § 1.495(b)(2). The payment of the surcharge set forth in § 1.492(e) is required as a condition for accepting the oath or declaration later than thirty (30) months after the priority date. The payment of the processing fee set forth in § 1.492(f) is required for acceptance of an English translation later than thirty (30) months after the priority date. Failure to comply with these requirements will result in abandonment of the application. The provisions of § 1.136 apply to the period which is set. Notice of Jan. 3, 1993, 1147 O.G. 29 to 40.

☒ **Assertion of Small Entity Status**

☒ **Applicant hereby asserts status as a small entity under 37 C.F.R. § 1.27.**

NOTE: 37 C.F.R. § 1.27(c) deals with the assertion of small entity status, whether by a written specific declaration thereof or by payment as a small entity of the basic filing fee or the fee for the entry into the national phase as states:

"(c) Assertion of small entity status. Any party (person, small business concern or nonprofit organization) should make a determination, pursuant to paragraph (f) of this section, of entitlement to be accorded small entity status based on the definitions set forth in paragraph (a) of this section, and must, in order to establish small entity status for the purpose of paying small entity fees, actually make an assertion of entitlement to small entity status, in the manner set forth in paragraphs (c)(1) or (c)(3) of this section, in the application or patent in which such small entity fees are to be paid.

(1) Assertion by writing. Small entity status may be established by a written assertion of entitlement to small entity status. A written assertion must:

- (i) Be clearly identifiable;
- (ii) Be signed (see paragraph (c)(2) of this section); and
- (iii) Convey the concept of entitlement to small entity status, such as by stating that applicant is a small entity, or that small entity status is entitled to be asserted for the application or patent. While no specific words or wording are required to assert small entity status, the intent to assert small entity status must be clearly indicated in order to comply with the assertion requirement.

(2) Parties who can sign and file the written assertion. The written assertion can be signed by:

- (i) One of the parties identified in §§ 1.33(b) (e.g., an attorney or agent registered with the Office), §§ 3.73(b) of this chapter notwithstanding, who can also file the written assertion;
- (ii) At least one of the individuals identified as an inventor (even though a §§ 1.63 executed oath or declaration has not been submitted), notwithstanding §§ 1.33(b)(4), who can also file the written assertion pursuant to the exception under §§ 1.33(b) of this part; or
- (iii) An assignee of an undivided part interest, notwithstanding §§ 1.33(b)(3) and 3.73(b) of this chapter, but the partial assignee cannot file the assertion without resort to a party identified under §§ 1.33(b) of this part.

(3) Assertion by payment of the small entity basic filing or basic national fee. The payment, by any party, of the exact amount of one of the small entity basic filing fees set forth in §§ 1.16(a), (f), (g), (h), or (k), or one of the small entity basic national fees set forth in §§ 1.492(a)(1), (a)(2), (a)(3), (a)(4), or (a)(5), will be treated as a written assertion of entitlement to small entity status even if the type of basic filing or basic national fee is inadvertently selected in error.

(i) If the Office accords small entity status based on payment of a small entity basic filing or basic national fee under paragraph (c)(3) of this section that is not applicable to that application, any balance of the small entity fee that is applicable to that application will be due along with the appropriate surcharge set forth in §§ 1.16(e), or §§ 1.16(f).

(ii) The payment of any small entity fee other than those set forth in paragraph (c)(3) of this section (whether in the exact fee amount or not) will not be treated as a written assertion of entitlement to small entity status and will not be sufficient to establish small entity status in an application or a patent."

3. ☒ A copy of the International application as filed (35 U.S.C. § 371(c)(2)):

NOTE: Section 1.495 (b) was amended to require that the basic national fee and a copy of the international application must be filed with the Office by 30 months from the priority date to avoid abandonment. "The International Bureau normally provides the copy of the international application to the Office in accordance with PCT Article 20. At the same time, the International Bureau notifies applicant of the communication to the Office. In accordance with PCT Rule 47.1, that notice shall be accepted by all designated offices as conclusive evidence that the communication has duly taken place. Thus, if the applicant desires to enter the national stage, the applicant normally need only check to be sure the notice from the International Bureau has been received and then pay the basic national fee by 30 months from the priority date." Notice of Jan. 7, 1993, 1147 O.G. 29 to 40, at 35-36. See item 14c below.

- a. ☐ is transmitted herewith.
- b. ☒ is not required, as the application was filed with the United States Receiving Office.
- c. ☐ has been transmitted
 - i. ☐ by the International Bureau.

Date of mailing of the application (from form PCT/1B/308):

- ii. ☐ by applicant on _____. (Date)

4. ☒ A translation of the International application into the English language (35 U.S.C. § 371(c)(2)):

- a. ☐ is transmitted herewith.
- b. ☒ is not required as the application was filed in English.
- c. ☐ was previously transmitted by applicant on _____. (Date)
- d. ☐ will follow.

5. ☒ Amendments to the claims of the International application under PCT Article 19 (35 U.S.C. § 371(c)(3)):

NOTE: The Notice of January 7, 1993 points out that 37 C.F.R. § 1.495(a) was amended to clarify the existing and continuing practice that PCT Article 19 amendments must be submitted by 30 months from the priority date and this deadline may not be extended. The Notice further advises that: "The failure to do so will not result in loss of the subject matter of the PCT Article 19 amendments. Applicant may submit that subject matter in a preliminary amendment filed under section 1.121. In many cases, filing an amendment under section 1.121 is preferable since grammatical or idiomatic errors may be corrected." 1147 O.G. 29-40, at 36.

- a. ☐ are transmitted herewith.
b. ☐ have been transmitted
i. ☐ by the International Bureau.

Date of mailing of the amendment (from form PCT/1B/308):

- ii. ☐ by applicant on _____. (Date)
c. ☒ have not been transmitted as
i. ☒ applicant chose not to make amendments under PCT Article 19.
Date of mailing of Search Report (from form PCT/ISA/210.):
7 September 2000
ii. ☐ the time limit for the submission of amendments has not yet expired. The amendments or a statement that amendments have not been made will be transmitted before the expiration of the time limit under PCT Rule 46.1.

6. ☒ A translation of the amendments to the claims under PCT Article 19 (38 U.S.C. § 371(c)(3)):

- a. ☐ is transmitted herewith.
b. ☐ is not required as the amendments were made in the English language.
c. ☒ has not been transmitted for reasons indicated at point 5(c) above.

7. ☒ A copy of the international examination report (PCT/IPEA/409)

- ☐ is transmitted herewith.
☒ is not required as the application was filed with the United States Receiving Office.

8. ☒ Annex(es) to the international preliminary examination report

- a. ☐ is/are transmitted herewith.
b. ☒ is/are not required as the application was filed with the United States Receiving Office.

9. ☒ A translation of the annexes to the international preliminary examination report

- a. ☐ is transmitted herewith.
b. ☒ is not required as the annexes are in the English language.

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10. ☒ An oath or declaration of the inventor (35 U.S.C. § 371(c)(4)) complying with 35 U.S.C. § 115
- a. ☐ was previously submitted by applicant on _____. (Date)
 - b. ☒ is submitted herewith, and such oath or declaration
 - i. ☒ is attached to the application.
 - ii. ☐ identifies the application and any amendments under PCT Article 19 that were transmitted as stated in points 3(b) or 3(c) and 5(b); and states that they were reviewed by the inventor as required by 37 C.F.R. § 1.70.
 - c. ☐ will follow.

II. Other document(s) or information included:

11. ☒ An International Search Report (PCT/ISA/210) or Declaration under PCT Article 17(2)(a):
- a. ☐ is transmitted herewith.
 - b. ☐ has been transmitted by the International Bureau.
Date of mailing (from form PCT/IB/308): _____
 - c. ☒ is not required, as the application was searched by the United States International Searching Authority.
 - d. ☐ will be transmitted promptly upon request.
 - e. ☐ has been submitted by applicant on _____. (Date)
12. ☒ An Information Disclosure Statement under 37 C.F.R. §§ 1.97 and 1.98:
- a. ☐ is transmitted herewith.

Also transmitted herewith is/are:

- ☐ Form PTO-1449 (PTO/SB/08A and 08B).
 - ☐ Copies of citations listed.
 - b. ☒ will be transmitted within THREE MONTHS of the date of submission of requirements under 35 U.S.C. § 371(c).
 - c. ☐ was previously submitted by applicant on _____. (Date)
13. ☒ An assignment document is transmitted herewith for recording.
- A separate ☐ "COVER SHEET FOR ASSIGNMENT (DOCUMENT) ACCOMPANYING NEW PATENT APPLICATION" or ☒ FORM PTO 1595 is also attached.
- New England Biolabs, Inc.
- _____

14. ☐ Additional documents:

- a. ☐ Copy of request (PCT/RO/101)
- b. ☐ International Publication No. _____
 - i. ☐ Specification, claims and drawing
 - ii. ☐ Front page only
- c. ☐ Preliminary amendment (37 C.F.R. § 1.121)
- d. ☐ Other

15. ☐ The above checked items are being transmitted

- a. ☐ before 30 months from any claimed priority date.
- b. ☐ after 30 months.

16. ☐ Certain requirements under 35 U.S.C. § 371 were previously submitted by the applicant on _____, namely:

AUTHORIZATION TO CHARGE ADDITIONAL FEES

WARNING: Accurately count claims, especially multiple dependant claims, to avoid unexpected high charges if extra claims are authorized.

NOTE: "A written request may be submitted in an application that is an authorization to treat any concurrent or future reply, requiring a petition for an extension of time under this paragraph for its timely submission, as incorporating a petition for extension of time for the appropriate length of time. An authorization to charge all required fees, fees under § 1.17, or all required extension of time fees will be treated as a constructive petition for an extension of time in any concurrent or future reply requiring a petition for an extension of time under this paragraph for its timely submission. Submission of the fee set forth in § 1.17(a) will also be treated as a constructive petition for an extension of time in any concurrent reply requiring a petition for an extension of time under this paragraph for its timely submission." 37 C.F.R. § 1.136(a)(3).

NOTE: "Amounts of twenty-five dollars or less will not be returned unless specifically requested within a reasonable time, nor will the payer be notified of such amounts; amounts over twenty-five dollars may be returned by check or, if requested, by credit to a deposit account." 37 C.F.R. § 1.26(a).

☒ Please charge, in the manner authorized above, the following additional fees that may be required by this paper and during the entire pendency of this application:

- ☒ 37 C.F.R. § 1.492(a)(1), (2), (3), and (4) (filing fees)

WARNING: Because failure to pay the national fee within 30 months without extension (37 C.F.R. § 1.495(b)(2)) results in abandonment of the application, it would be best to always check the above box.

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SURFACE DISPLAY OF SELENOCYSTEINE-CONTAINING PEPTIDES

BACKGROUND OF THE INVENTION

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The fusion of peptides to the coat proteins of amplifiable genetic particles, e.g., phage, is a widely used method for screening combinatorial libraries of peptides (Rodi and Malowski, *Curr. Opin. Biotechnol.*, 10:87-93 (1999); Wilson and Finlay, *Canadian Journal of Microbiology*, 44:313-329 (1998)). One common approach is to express random sequences at the N-terminus of the bacteriophage M13 coat protein pIII, resulting in library complexities of up to 10^9 different clones. Selection is achieved by performing multiple rounds of target binding (panning), elution and amplification. Each round of panning enriches the pool of clones in favor of the tightest-binding ligands. Because each phage particle contains both the displayed peptide and the DNA encoding it, the selected peptides can be readily identified by DNA sequencing. Despite its utility and convenience, *in-vivo* biological expression limits library diversity to combinations of twenty of the naturally occurring amino acids, linked by peptide bonds.

This problem can be partially circumvented by taking advantage of the enormous potential chemical diversity of synthetic combinatorial libraries. A vast body of work has been carried out with libraries consisting of systematic variations of peptides (Geysen, et al., *Proc. Natl. Acad. Sci.*

USA, 81:3998-4002 (1984); Houghten, et al., *Nature* 354:84-86 (1991), Lam, et al., *Nature*, 354: 82-84 (1991)), peptide analogues (Figliozzi, et al., *Methods Enzymol.*, 267:437-447 (1996), and small molecules (Bunin, et al., *Methods Enzymol.*, 267:448-465 (1996), and an entire industry has been built around this type of combinatorial chemistry. While libraries well in excess of 10^{18} different molecules (equivalent to 1 μ mol of material if one molecule of each variant is present) can be synthesized, the identification of which molecules bind to a given target from such a vast pool is problematic. Libraries are typically synthesized in spatially addressable form, e.g., grids of pins or wells each containing one compound (Geysen, *supra*), or tethered to macromolecular beads containing a chemical tag which specifically identifies the attached compound (Lam, et al., *supra*). Ligand identification thus limits the size of chemically synthesized libraries to a practical upper limit of 10^4 - 10^6 different molecules. Unlike biosynthetic libraries such as phage display peptide libraries, however, chemically synthesized libraries are not limited to a small subset of potential functional diversity.

The functional diversity of phage displayed peptide libraries can be increased by specifically chemically modifying the library prior to each round of panning. Phage libraries with enzymatically phosphorylated tyrosine residues have been constructed to map protein kinase and SH2 domain recognition sequences (Dente, et al., *Journal of Molecular*

Biology, 269:694-703 (1997); Schmitz, et al., *J. Mol. Biol.*, 260:664-677 (1996)). Phage libraries have also been biotinylated at specific lysine residues during *in vivo* phage morphogenesis, but this method requires a specific 66-residue biotinylation motif (Stolz, et al., *FEBS Lett.*, 440:213-217 (1998)). Both of these methods require defined flanking sequence, and the incorporated modification cannot be altered. Therefore neither are generally applicable to incorporation of any desired chemical functionality in the context of a randomized amino acid sequence. For example, there are no methods for specifically modifying displayed tyrosine with other chemical moieties while protecting endogenous tyrosine residues elsewhere on the phage coat. The side chains of lysine and cysteine are reactive, but small-molecule reagents are likely to target residues within the native coat protein in addition to the displayed peptide. A new type of phage library, with a unique site available for a broad range of chemical modifications, is therefore needed.

To maintain the essential amplification and selection techniques of phage display, the existing bacterial genetic machinery should be employed to incorporate the unique reactive site into the displayed peptide. A method in which a non-native residue is incorporated into a phage-displayed protein by native chemical ligation (Dwyer, et al., *Chem. Biol.*, 7:263-274 (2000)) could in principle be used to incorporate a unique reactive site, but this method requires that the non-native residue be incorporated within a synthetic peptide

sequence, which is then chemically ligated onto a phage
displayed polypeptide. As a result, the residues flanking the
potential modification site are not encoded on the phage
genome, severing the link between displayed sequence and
DNA sequence.

SUMMARY OF THE INVENTION

In accordance with the present invention, the power of
in vivo biomolecular amplification with the unlimited diversity
of small molecule chemistry is united in selenopeptide phage
display. The naturally-occurring amino acid selenocysteine
(Sec) is incorporated uniquely and specifically in the context of
a polypeptide displayed on the surface of an amplifiable
genetic particle (phage, polysome, cell or spore) in response
to incorporation signals engineered in the encoding DNA. In
addition to conferring the unique activities of the selenol
group to the chemistry of the displayed peptide, Sec also
provides a unique handle for specific chemical modification of
the displayed peptide. In addition to increasing the palette of
available residues in a random peptide library to 21
possibilities, the present invention also provides a means of
tethering virtually any desired chemical functionality to the
incorporated Sec. Applications include, but are not limited to,
pre-modifying a random peptide library with enzyme
substrate analogs or inhibitors prior to panning for higher-
affinity inhibitors, as well as selection/evolution of displayed
enzyme specificity by catalytic selection using a substrate

tethered to the same particle via an incorporated selenocysteine. Additionally, the coupling of Sec incorporation to phage plaque formation provides a rapid nonradioactive assay for DNA sequence requirements for efficient Sec incorporation.

DESCRIPTION OF THE DRAWINGS

Figure 1 - Biosynthetic pathway for cotranslational selenocysteine incorporation. Sel C, the opal codon-specific Sec tRNA, is first charged with serine. ATP-dependent SelD catalysis transforms environmental selenite to an activated Se-phosphate species. This species is utilized by Sela to displace the serine hydroxyl with a selenol moiety, forming a Sec-charged tRNA that recognizes the UGA opal codon. In the presence of GTP, the SelB elongation factor effects Sec translation by binding both the Sec-tRNA and the mRNA SECIS.

Figure 2 - The *E. coli* formate dehydrogenase (fdh) SECIS, with permissible mutations as reported in Heider, et al., *EMBO J.*, 11:3759-3766 (1992); Klug, et al., *Proc. Natl. Acad. Sci. USA* 94:6676-6681 (1997); Liu, et al., *Nucleic Acids Res.*, 26:896-902 (1998).

Figure 3 - N-terminal protein sequencing of the fusion of the fdh SECIS with the maltose binding protein. HPLC PTH

analysis is displayed in subtractive mode. The expected sequence was SARVSecHGPSV.

Figure 3A - Cycle 3 subtracted from cycle 4.

Figure 3B - Cycle 4 subtracted from cycle 5.

5 Figure 3C - Cycle 5 subtracted from cycle 6. S = serine; S' = dehydroalanine (expected acid breakdown product of Sec).

10 Figure 4 - The randomized SECIS library inserts expressed as M13 pIII fusions. N = A, G, C or U. K = G or U. Permissible mutations are based on the *E. coli* formate dehydrogenase (fdh) SECIS as reported in Liu, et al., *supra*. The position immediately downstream from the UGA codon is fully randomized in the TGAN library, and is fixed as U in the
15 TGAT library. (SEQ ID NO:1)

20 Figure 5 - Plating results showing Se-dependent and -independent growth of phage clones TGAT-13, TGAT-10 and TGAN-8 (sequences in Figures 9 and 10). Left column: no supplemental Se in plating medium. Right column: 2 μ M sodium selenite added to plating medium.

25 Figure 6 - Clone TGAT-6 plaque count and size as a function of supplemental sodium selenite concentration in plating medium. Visible plaques were counted without magnification, and plaque diameter was measured under 7-fold magnification. Error bars represent ± 1 standard

deviation. Averages were based on triplicate platings, with ten plaques measured per plate.

Figure 7 - Immunoblots of biotinylated phage, probed
5 with HRP-conjugated anti-biotin antibody (NEB) and visualized
by chemiluminescence. Phage (10^{11} pfu) were diluted in 150
mM NaCl, 50 mM glycine-HCl (pH 2.5). Iodoacetyl-LC-Biotin (I-
Bt) in DMF was added at 5% v/v to the indicated final
10 concentration, and the reactions were incubated in the dark
at room temperature. MW: biotinylated molecular weight
markers.

Figure 7A - Phage samples (peptide sequences in Figure
9), treated with I-Bt for 30 min.

Figure 7B - Sec-1 samples, treated with the indicated
15 concentration of iodoacetamide for 1 h at room temperature,
followed by 50 μ M I-Bt for 20 min.

Figure 8 - Immunoblots showing specific chemical
modification of phage displaying selenopeptides. Individual
20 library clones from the TGAN library (A) or TGAT library (B)
were amplified with or without 2 μ M supplemental sodium
selenite as indicated. Phage were modified as described in
Example III. *Amplification of clone TGAT-1 in
unsupplemented medium resulted in a TGA \rightarrow TGG point
25 mutation.

Figure 9 - Selected library TGAN clones. TGAN-1 was
expressed as a pMal-pIII fusion, and tryptophan incorporation

was verified by N-terminal sequencing. ^aop: opal
suppression: Sec or W, depending on Se availability. ^bTGAN-
7 production was Se-enhanced; Se supplementation yielded
larger (3-4x diameter) and more (10x) plaques. (SEQ ID NO:2
5 through SEQ ID NO:14).

Figure 10 - Selected TGAT library clones. TGAT-13
carried a T→C point mutation within the opal codon. (SEQ ID
NO:15 through SEQ ID NO:27).

Figure 11 - Schematic of selenosulfide-constrained
heptapeptide library displayed as an N-terminal fusion to pIII
of M13. The randomized sequence is flanked by an upstream
cysteine (C) residue and a downstream selenocysteine (Sec)
15 residue, which spontaneously oxidize to yield a redox-stable
selenosulfide cross-link. Selenocysteine is encoded by the
opal codon UAG with a SECIS immediately downstream. Each
randomized residue is encoded by NNK, where N is an
equimolar mixture of G, A, U, C; and K is equimolar U and G.
20 (SEQ ID NO:40 and SEQ ID NO:41)

Figure 12 - Phage ELISA showing binding of the
indicated linear (Ser-Ser), disulfide constrained (Cys-Cys) and
selenosulfide constrained (Cys-Sec) sequences to
streptavidin, in the presence and absence of 10 mM
25 dithiothreitol (DTT). Purified phage displaying the indicated
constrained or unconstrained peptide sequence were added
in the indicated amounts (pfu, plaque forming units).

Following washing, bound phage were detected with anti-M13 antibody conjugated to horseradish peroxidase. Following development with ABTS/H₂O₂ in citrate buffer, plates were read in an ELISA plate reader at 405 nm.

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DETAILED DESCRIPTION OF THE INVENTION

The present invention which describes a method for biosynthetic incorporation of a unique reactive site takes advantage of the naturally occurring amino acid selenocysteine (Sec). The potential modifications of Sec derive from its unique chemical properties. The pK_a of Sec is 5.2, compared to 8.1 for Cys, so that at pH 6-7, nucleophilic substitution reactions can specifically alkylate Sec, but not Cys residues (Gorlatov and Stadtman, et al., *Proc. Natl. Acad. Sci. USA* 95:8520-8525 (1998)). The formation of stable sulfide-selenide cross-links (Pegoraro, et al., *J. Mol. Biol.* 284:779-792 (198)) also permits covalent Sec modification by thiol reagents.

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Incorporation of selenocysteine involves harnessing the existing biosynthetic mechanism. Eubacterial Selenocysteine (Sec) incorporation, as depicted in Figure 1, has been well characterized and requires the constitutively expressed *selA*, *selB*, *selC* and *selD* gene products (Böck, et al., *Mol. Microbiol.*, 5:515-520 (1991)). Sec is encoded by the TGA opal stop codon (Zinoni, et al., *Proc. Natl. Acad. Sci. USA* 84:3156-3160 (1987)), which is suppressed in the presence of a specific

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downstream hairpin structure termed the Selenocysteine Insertion Sequence (SECIS). Sec is incorporated via a unique tRNA species, the *se/C* gene product, which is initially aminoacylated with serine by seryl-tRNA synthetase. The loaded serine is converted to selenocysteine by the *se/A* gene product, using a selenium phosphate donor synthesized by the *se/D* gene product. Translation by the resulting Sec-tRNA^{Sec} is mediated by the *se/B* product, an analog of (the Elongation Factor EF-Tu which simultaneously recognizes Sec-tRNA^{Sec} and the SECIS. The incorporation mechanism in Eukarya and Archaea is nearly identical (Stadtman, *Ann. Rev. Biochem.* 65:83-100 (1996)).

The mRNA requirements for *E. coli* Sec incorporation, summarized in Figure 2, indicate that the minimal SECIS consists of a short hairpin sequence with fixed nucleotides, located exactly 11 bases downstream from the UGA stop codon. Considerable nucleotide flexibility is allowed in this intervening sequence, permitting incorporation of selenocysteine within a randomized stretch of amino acids.

The present invention comprises three components:

(a) an expression system for display of heterologous peptide and protein sequences on the surface of amplifiable genetic particles (bacteriophage, virus, polysome, cells, spores, etc.) as fusions to surface proteins;

(b) a UGA opal codon at the position in the displayed polypeptide where selenocysteine is to be incorporated; and

(c) a minimal SECIS at the proper distance
5 downstream from the UGA codon to direct Sec incorporation, incorporated so as not to interrupt the reading frame of the displayed polypeptide-surface protein fusion.

It is demonstrated in the Examples herein that
10 selenopeptide libraries displayed on the surface of bacteriophage can be generated using an adaptation of standard phage display methods. The evidence for selenocysteine incorporation is described in Sandman, et al.,
15 *J. Am. Chem. Soc.*, 122:960-961 (2000) and Sandman and Noren, *Nucleic Acids Res.* 28:755-761 (2000). Specifically, all of the TGAT library clones assumed to display exclusively selenopeptides formed plaques only in the presence of supplemental selenium. N-terminal sequencing of Maltose Binding Protein fusions revealed dehydroalanine, and not Trp,
20 in several putative Sec-inserting clones. The chemical modification of the phage samples believed to contain Sec was consistent with selenium reactivity, with nucleophilic substitution readily occurring at acidic pH, where Cys is expected to be unreactive. Finally, the occurrence of clones
25 encoding Cys proximal to the TGA codon also implicates Sec incorporation, since sulfide-selenide bridging would stabilize the otherwise unpaired Cys.

In accordance with the presentation, the molecular diversity of displayed peptides can now include twenty-one amino acids instead of the traditional twenty, but since the twenty-first amino acid can be specifically chemically modified, any desired functionality can be appended prior to each round of panning. Small libraries of appended functionalities may be screened by modifying the peptide libraries in separate and spatially addressable reaction vessels. Enzyme inhibitors may be identified by modifying Sec with substrate or transition state analogs and panning the resulting modified peptide libraries against enzymes. By tethering a known low-affinity inhibitor to a random peptide library, flanking residues which increase the overall affinity of the inhibitor-peptide chimera can be selected by standard phage panning methods. This concept of iterative ligand assembly has recently been demonstrated with small-molecule libraries (Maly, et al., *Proc. Natl. Acad. Sci. USA*, 97:2419-2424 (2000)), but the present invention extends this idea to take advantage of the vastly higher-complexity libraries allowed by surface display methods. The linkage of cytotoxic agents to the Sec residue, for example, may facilitate the discovery of peptide-drug complexes that are taken up into specific cell types. Particles simultaneously displaying enzyme libraries and substrate-derivatized selenopeptides may be screened for enzymes with enhanced activity or altered specificity. The covalent linkage of substrates to displayed peptides permits the rigorous reaction and selection conditions that might otherwise disrupt the noncovalent interactions utilized in

recent examples of phage-mediated enzyme evolution.(Demartis, et al., *J. Mol. Biol.*, 286:617-633 (1999); Pedersen, et al., *Proc. Natl. Acad. Sci, USA* 95:10523-10528 (1998)).

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The present invention also provides a useful tool for further study of the requirements for selenocysteine incorporation. By coupling plaque formation to selenocysteine incorporation it is possible to screen thousands of sequences at once, using a simple, nonradioactive visual readout that specifically indicates Sec incorporation rather than general opal suppression. Previous studies of prokaryotic SECIS requirements suggested that certain elements of the mRNA structure were essential, whereas others could be changed without affecting opal codon readthrough (Liu, et al., *supra*). In accordance with the methods of the present invention, it was rapidly determined that the first nucleotide downstream of TGA strongly influences opal suppression in *E. coli*, with purines or CTG promoting a dual-pathway approach in which Trp insertion is always possible, and Sec insertion occurs if Se is available. TGA-pyrimidine sequences, on the other hand, only permit the inefficient cotranslational Sec insertion pathway, thereby occasionally allowing opal codon mutants to dominate a culture. The apparent stabilization of unpaired Cys residues by Sec overrides these rules, so that an adjacent unpaired Cys will strongly favor the Sec insertion pathway, regardless of the downstream nucleotide. This application has important implications for heterologous

expression of mammalian selenoproteins in prokaryotic systems (Arner, et al., *J. Mol. Biol.* 292:1003-1016 (1999)): the present invention can be used to optimize the expression level of virtually any selenoprotein.

The present invention is further illustrated by the following Examples. These Examples are provided to aid in the understanding of the invention and are not construed to be a limitation thereof.

The references cited above and below are herein incorporated by reference.

EXAMPLE I

EXPRESSION OF NATIVE *E. COLI* *fdh* SEQUENCES AS M13 pIII FUSION

As a control, the native *E. coli* formate dehydrogenase (*fdh*) SECIS (Figure 2, amino acid sequence Ser-Ala-Arg-Val-Sec-His-Gly-Pro (SEQ ID NO:28)) was cloned into M13KE, an M13mp19 derivative designed with *Acc65I* and *EagI* sites for pentavalent N-terminal pIII expression (Zwick, et al., *Analytical Biochemistry*, 264:87-97 (1998)). The following oligonucleotides were synthesized by the phosphoramidite method by the Organic Synthesis Division of New England Biolabs, Inc. (Beverly, MA). *Acc65I* and *EagI* restriction sites are indicated in **bold**.

fdh SECIS control oligonucleotide:

5'-CATGTTTT**CGGCCG**TACCGACCGATTGGTGCAGACCTGCAACCGA
TGGGCCCGTGTTCAGACACGAGCGCTAGAGTGAGAATAGAA**AGGTACC**
CGGGCATG-3' (SEQ ID NO:29)

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Duplex extension primer (New England Biolabs, (Beverly, MA)
product #8101): 5'-CATGCCCC**GGTACCTTT**CTATTCTC-3' (SEQ
ID NO:30)

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The fdh SECIS control oligonucleotide was synthesized,
gel-purified, and annealed to the duplex extension primer.

The duplex was extended with dNTPs and Klenow fragment,
digested with Acc65I and EagI, gel-purified and ligated into
Acc65I/EagI digested phage cloning vector M13KE. The

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ligation products were electroporated into *E. coli* ER2537 (*F'*
lacI^qΔ(lacZ)M15 proA⁺B⁺/fhuA2 supE thiΔ(lac-proAB)Δ(hsdMS-

*mcrB)*5 and plated with 100 μL of a late log-phase ER2537
culture in 3 mL of agarose top on LB agar plates with 210 μM
IPTG and 98 μM Xgal. The agarose top contained 10 g/L

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tryptone (Difco, Detroit, MI), 5 g/L yeast extract (Difco, Detroit,
MI), 86 mM sodium chloride, 5 mM magnesium chloride, and 7
g/L agarose (American Bioanalytical, Natick, MA)

supplemented with 2 μM sodium selenite. The M13KE vector
carries the lacZα fragment, resulting in characteristic blue

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plaques when plated with an α-complementing strain on X-gal
medium. After a 16 h 37°C incubation, blue plaques were
selected and the individual clones were amplified in early log-
phase cultures of ER2537 supplemented with 2 μM sodium

selenite. Sequencing templates were prepared by ethanol precipitation of phage DNA from 4 M sodium iodide (Wilson, *Biotechniques* 15:414-416, 418-420, and 420 (1993)). Phage clones were stored at 4°C in a 150 mM Tris pH 7.4, 50 mM sodium chloride, 100 µM DTT buffer with 0.02% sodium azide. Automated DNA sequencing was performed on a PE-ABD 377 or 373 instrument using Dye-Deoxy™ terminator chemistry (PE Applied Biosystems, Foster City, CA) with the -96 gIII sequencing primer (New England Biolabs, Inc. (Beverly, MA) product #1259, 5'-CCCTCATAGTTAGCGTA ACG-3' (SEQ ID NO:31)), and yielded the expected sequence (designated Sec-1).

pMal-pIII fusion protein expression.

In order to obtain sufficient quantity of material for confirmation of Sec incorporation by N-terminal protein sequencing, the pMal-pIII shuttle vector (Zwick, et al., *supra*) was employed to overexpress and purify the Sec-1 peptide sequence as a fusion to the N-terminus of maltose binding protein (MBP). The resulting construct contains a pIII leader sequence to direct the fusion to the periplasm, resulting in the N-terminus of the MBP fusion being identical to that of the phage-displayed fdh sequence. The digested and gel-purified fdh SECIS insert was ligated into Acc65I/EagI digested pMal-pIII protein expression vector. The ligation products were electroporated into ER2537, plated on LB with 100 µg/mL ampicillin, and analyzed by restriction mapping and

automated DNA sequencing. The pMal-pIII fusion proteins were expressed in ER2537 and purified as previously described (Zwick, et al., *supra*). For N-terminal protein sequencing, proteins were subjected to electrophoresis and electroblotted according to the procedure of Matsudaira (Matsudaira, *J. Biol. Chem.*, 262:10035-10038 (1987)), with modifications as previously described (Looney, et al., *Gene* 80:193-208 (1989); Waite-Rees, et al., *J. Bacteriol.* 173:5207-5219 (1991)). The membrane was stained with Coomassie blue R-250 and the protein band of approximately 46 kDa was excised and subjected to sequential Edman degradation on a PE-Biosystems (Foster City, CA) 494A Protein/Peptide Sequencer using standard gas-phase cycles (Waite-Rees, *supra*). The results (Figure 3) showed the expected Sec-1 N-terminus, SARVXHGPSV (SEQ ID NO:32), with X assumed to be Sec. The acid breakdown product of a Sec residue, generated by acid-catalyzed β -elimination, should be the same as Cys or Ser residues in that all produce dehydroalanine (S'). The DTT adduct of the dehydroalanine PTH was observed at the position corresponding to the TGA codon (cycle 5). Ser also produces this adduct, but cycles 1 and 9, and not cycle 5, also showed a parent Ser peak. No significant amount of Trp-PTH (< 200 fmol) was observed in this cycle, eliminating the possibility of endogenous Trp-inserting opal suppression.

EXAMPLE II

CONSTRUCTION AND CHARACTERIZATION OF TGAN AND TGAT LIBRARIES

5 Based on the reported minimal SECIS requirements
(Figure 2) (Liu, et al., *supra*), a library consisting of the SECIS
element with four upstream and three downstream
randomized codons, and a minimal mRNA SECIS (TGAN library,
10 Figure 4), was prepared using the same cloning strategy
described in Example I. The TGAN library oligo sequence was
as follows, with *Acc*65I and *Eag*I restriction sites in **bold**; M=A
or C; N=A, C, T or G.

15 5'-CATGTTT**CGGCCG**ATTGGTGCAGACCTGCAACCGAMNNMNNM
NNTCAMNNMNNMNNMNNAGAGTGAGAATAGAAAG**GGTACCCGGG**-3'
(SEQ ID NO:33)

20 After duplex extension and restriction digestion, the
resulting insert was ligated into M13KE, an M13mp19
derivative designed with *Acc*65I and *Eag*I sites for
pentavalent N-terminal pIII expression (Zwick, et al., *supra*).
This vector also carries the lacZ α fragment, resulting in
characteristic blue plaques when plated with an α -
25 complementing strain on X-gal medium. The sequences of
selected clones are shown in Figure 9. Although the
immediate downstream nucleotide was fully randomized in
the TGAN library insert, the majority (74%) of the resulting
phage clones possessed a downstream purine. One-third of

the displayed peptides in the TGAN library possessed an unpaired Cys residue, which corresponds to 4.8% of the total randomized amino acids. Based on random codon usage, the calculated expected frequency for Cys is 3.1%, whereas the typically observed Cys frequency using this phage display system is less than 0.5%. Because M13 proteins are exported to the periplasm, the pairing of the eight Cys residues in M13 pIII into four disulfide bonds (Holliger and Riechmann, *Structure* 5:265-275 (1997)) could be disrupted by a single unpaired Cys within the displayed peptide. This phenomenon would likely not be observed with a cytoplasmically-expressed peptide library.

A second library (TGAT library, Figure 4) was also constructed in which the nucleotide immediately downstream from the UGA codon was fixed as U (T in the DNA) in order to prevent endogenous tryptophan-inserting opal suppression, which is enhanced by downstream purines and the trinucleotide CUG (Miller and Albertini, *J. Mol. Biol.* 164:59-71 (1983)). The TGAT library was constructed as described above using the library oligonucleotide 5'- 5'-CATGTTT**CGGCC** **GATTGGTGCAGACCTGCAACCGAMNNMNNMNNATCAMNNMNNMNN** **MNNAGAGTGAGAATAGAAAAGGTACCCGGG**-3' (SEQ ID NO:34), where the EagI and Acc65I sites are indicated in **bold** (M=A or C; N=A, C, T or G). The electroporation and plating of the library ligation products resulted in small plaques, with about ten times more plaques forming in the presence of 2 μ M supplemental sodium selenite as compared to

unsupplemented medium. Individual plaques were amplified for further analysis, with representative sequences shown in Figure 10. The growth of all of the TGA-containing clones was strictly selenium-dependent, with plaques appearing only in the presence of 1-2 μ M supplemental sodium selenite. M13KE phage growth, by contrast, was selenium-independent over a range of 0-4.5 μ M supplemental sodium selenite. As with the TGAN library, single Cys residues occurred in the TGAT library at a higher than normal frequency of 4.3% of all random amino acids. The TGAT library also had occasional (<10% frequency) mutations within the opal codon, such as the point mutation in clone TGAT-13 (Figure 10), which converted the TGA opal codon to the CGA Arg codon.

Selenium dependency of phage growth.

To assess the selenium dependency of phage production, phage samples were plated in media with or without 2 μ M supplemental sodium selenite. Individual phage clones were diluted in LB and combined with 200 μ L of a late log-phase ER2537 or ER2738 culture. After a 5 min incubation at room temperature, the bacteria and phage were combined with 3 mL of agarose top, with or without supplemental 2 μ M sodium selenite, and plated on LB agar plates with IPTG and Xgal. After a 16 h 37°C incubation the plates were inspected for the presence of blue plaques. Typical results are illustrated in Figure 5 and summarized in the third column of Figure 9 and Figure 10. Selenium-independent phage clones,

such as TGAT-13 and TGAN-8, produced plaques of equal count and size regardless of the media selenium concentration. In contrast, clone TGAT-10 and other Se-dependent phage only produced plaques in Se-supplemented medium. To further quantitate the strict selenium dependence of phage growth, Clone TGAT-6 (ASPTSecFKP) was plated with varying concentrations of supplemental sodium selenite in the medium. Figure 6 shows that the number and diameter of visible Clone 6 plaques increased in a selenium-dependent fashion from 0-3.2 μ M sodium selenite, with half-maximal plaque diameter at \sim 0.4 μ M selenite.

All of the clones with an immediate downstream purine grew in a Se-independent manner, with the exception of those containing a single Cys codon within the displayed peptide sequence, *e.g.*, TGAN-7, Figure 9. All of the clones with an opal codon immediately followed by a pyrimidine grew in a Se-dependent manner, with the exception of clone TGAN-10, which had a downstream CTG codon. All of the clones with mutated opal codons, such as TGAT-13, were Se-independent, as was M13KE phage without insert.

N-terminal sequencing.

To further analyze the displayed peptides, the pMal-pIII shuttle vector was employed (Zwick, et al., *supra*). This vector allows inserts from M13KE to be expressed as fusions to the N-terminus of maltose binding protein (MBP), with a pIII

leader sequence to direct the fusions to the periplasm. The
TGAN-1 peptide, in which the UGA codon is followed by an A,
was overexpressed and purified using this system.
N-terminal sequencing revealed mostly (>90%) Trp
5 incorporation at the TGA site, as expected from endogenous
Trp-inserting opal suppression favored in this sequence
context (Miller and Albertini, *supra*). This is fully consistent
with the observed selenium independence of this clone. By
contrast, the results of sequencing an MBP fusion with the
10 selenium-dependent clone TGAT-12 were comparable to data
obtained with the Sec-1 *E. coli fdh* SECIS insert (Figure 3),
consistent with Sec and not Trp insertion.

EXAMPLE III

CHEMICAL MODIFICATION OF SELENOPEPTIDE LIBRARIES

To rule out the possibility of Cys incorporation at the
20 TGA codon, and to demonstrate specific chemical modification
of the Sec residue in a displayed peptide, the chemical
reactivity of the *fdh* control phage clone Sec-1 (SARV-Sec-HGP)
was compared to that of clone Cys-1 (SARVLCNH (SEQ ID
NO:35)), which contains a single unpaired cysteine residue.
25 Phage samples were treated with iodoacetyl-LC-biotin (I-Bt,
Pierce), an electrophilic reagent which should specifically
target thiol or selenol groups with the enzyme cofactor biotin.
Phage (10^{10} pfu) in 150 mM sodium chloride, 50 mM glycine-
HCl (pH 2.5) were combined with 50 μ M iodoacetyl-LC-biotin in

dimethylformamide (5% v/v) and incubated in the dark at room temperature for 10 min. The reactions were quenched by the addition of SDS gel loading buffer with 42 mM DTT, and samples were promptly denatured at 100°C for 5 min and loaded on a 10-20% SDS-polyacrylamide gel. Immunoblotting was performed according to standard procedures, and the blots were probed with HRP-conjugated anti-biotin antibody (1:1000 dilution) or a mouse monoclonal anti-pIII antibody (Bio 101; 1:500 dilution) followed by an HRP-conjugated anti-mouse antibody. The blots were developed using the Phototope® Chemiluminescence kit (New England Biolabs, Inc., Beverly, MA).

Figure 7A shows the results of immunoblotting of the products. At both pH 2.5 and pH 8, the biotinylation was highly specific for the Sec residue. Biotinylation of Cys-1 was enhanced at pH 8, although the reaction remained highly selective (>10:1) for Sec. The biotinylation experiments confirm that, at acidic pH, the reactivity of a Sec residue in a pIII fusion greatly exceeds that of the eight paired Cys residues (Holliger and Riechmann, *supra*) in M13 pIII or of an unpaired Cys residue in the displayed peptide.

Presumably because of the stability of sulfide-selenide cross-links, the selenopeptide library contained clones with a single Cys residue at a much higher incidence than is normally seen in pIII libraries constructed in the M13KE system. To determine whether the putative sulfide-selenide bridging

inhibited Sec reactivity, phage clones TGAT-2 and TGAT-3 were modified with I-Bt; immunoblotting revealed that both samples were biotinylated to a similar extent as Sec-1 (Figure 7A). This result suggests that the sulfide-selenide cross-link is sufficiently reversible to allow trapping of the free selenide with an excess of electrophile.

To estimate the efficiency of chemical modification, Sec-1 phage was modified with iodoacetamide (I-Ac), and the remaining unmodified phage was then reacted with I-Bt and detected by immunoblotting (Figure 7B). Treatment for 1 h with 250 μ M I-Ac at pH 2.5 was sufficient to block the biotinylation reaction. Because the electrophilicities of I-Ac and I-Bt are essentially identical, this result suggests that modification with I-Bt under these conditions would go to completion. To assess the infectivity of the modified phage, the Sec-1 clone was treated for 1 h at room temperature at pH 5.2 with I-Ac or I-Bt. After quenching with two equivalents of β -mercaptoethanol to scavenge any unreacted I-X electrophile, the samples were diluted and plated, with no significant effect on the resulting plaque counts.

EXAMPLE IV

IDENTIFICATION OF SECIS REQUIREMENTS

The mRNA requirements for *E. coli* Sec incorporation were previously determined by cloning the *E. coli* formate dehydrogenase gene (*fdh*) with non-native SECIS variants

upstream of a β -galactosidase reporter gene, and then measuring either ^{75}Se incorporation by SDS-PAGE or β -galactosidase expression by a colorimetric assay (Chen, et al., *J. Biological Chemistry*, 268:23128-23131 (1993); Heider, et al., *EMBO J.*, 11:3759-3766 (1992); Liu, et al., *supra*; Zinoni, et al., *Proc. Natl. Acad. Sci. USA* 84:3156-3160 (1990). Although reporter gene expression is the more quantitative of the two approaches, it is a measure of TGA suppression but not necessarily Sec incorporation. Many *E. coli* strains possess endogenous opal suppression activity resulting in tryptophan (Trp) incorporation (Miller and Albertini, *supra*), suggesting that a portion of the reporter gene expression could have been independent of Sec incorporation.

The coupled phage display assay which comprises the present invention was utilized to further investigate the *E. Coli* SECIS requirements (Sandman and Noren, *supra*). The native M13 proteins do not contain Sec (Ebright, et al., *Gene* 114:81-83 (1992); van Wezenbeek and Schoenmakers, *supra*), and M13 phage infectivity requires expression of the coat protein pIII (Holliger and Riechmann, *supra*). The fusion of putative selenopeptides to the N-terminus of pIII therefore should couple phage plaque formation to opal suppression. Because of the relatively high level of protein synthesis required for plaque formation, it was anticipated that selenium-supplemented media would be required for selenopeptide phage display. Putative selenopeptide-pIII fusions could thus be identified based on the selenium

dependence of plaque formation, and this *in vivo* method could be used to identify critical SECIS elements from phage libraries. The utility of *in vitro* combinatorial methods was previously shown when RNA aptamer libraries were screened for SelB binding (Klug, et al., *supra*). The SECIS U₁₇ bulge, noted in Figure 2, was found to be required for SelB binding, which in turn is necessary for Sec incorporation. Whereas the aptamer method only revealed *in vitro* binding events, the phage display method provides a direct readout of prokaryotic Sec incorporation requirements *in vivo*.

In addition to selenium dependency of phage formation, Sec incorporation can also be assessed by modifying phage samples with readily detectable reagents as described in Example III. The pKa of Sec is 5.2, compared to 8.1 for cysteine (Cys), so that at pH 6-7, nucleophilic substitution reactions can specifically alkylate a Sec residue without modifying neighboring Cys residues (Gorlatov and Stadtman, *supra*). Example III describes a method to assay phage for selenopeptide display by treatment with an electrophilic iodoacetamido-biotin reagent, followed by detection of biotinylated phage with an anti-biotin antibody. Because the reactivity of Sec is unique from that of any other naturally occurring amino acid side chain, chemical reactivity is a more specific indicator of Sec than opal suppression.

Effect of media selenite on selenocysteine incorporation.

To explore the effect of sequence context on opal suppression, individual phage clones were amplified in media with or without supplemental 2 μ M selenite. The resulting phage was quantitated by plating diluted samples, and the phage DNA was sequenced. As a test of Sec incorporation in the displayed peptides, phage clones were treated with iodoacetyl-LC-biotin (I-Bt) as in Example III, and the level of biotinylation was assessed by immunoblotting. As controls, M13KE phage and clones displaying a single unpaired Cys (Cys-1, displayed peptide SARVLCNH (SEQ ID NO:35) or Sec (Sec-1, displayed peptide SARVSecHGP, corresponding to the *E. coli fdh* SECIS) were used.

Clones TGAN-12 and TGAN-8, both of which had a downstream purine, produced equivalent levels of phage in supplemented and unsupplemented media. Growth of both clones in media with selenite substantially enhanced the reactivity of the resulting phage (Figure 8A). Production of TGAN-7, which had a downstream purine and a single Cys, was enhanced 50-fold by supplemental Se. Clones with a downstream pyrimidine and a single Cys in the displayed peptide, such as TGAT-7 and TGAT-2, produced 1000-fold less phage without supplemental Se. The phage produced with supplemental Se from these clones had reactivity equal to that of the control Sec-1 phage (Figure 8B). Clones such as TGAT-1, which possessed a downstream pyrimidine and no

Cys in the displayed peptide, either had very low phage production in the absence of supplemental Se, or produced phage with opal codon mutations. Figure 8B shows that clone TGAT-1 was reactive when amplified with Se; the TGA → TGG mutant resulting from amplification without Se was unreactive. Occasionally, TGAT clones also developed opal codon point mutations during amplification with supplemental Se.

The expression of randomized SECIS elements as N-terminal fusions to M13 pIII couples phage production to opal suppression, providing a combinatorial approach to understanding cotranslational Sec insertion. If a sequence fails to produce phage, then it is assumed that there is no opal suppression. If phage is produced in a Se-dependent manner, the opal suppression is presumed to be Sec-inserting. Se-independent phage production can result from Trp insertion or from mutations within the opal codon.

In addition to the principal requirement for Sec incorporation, the opal codon with a downstream SECIS, the invention demonstrates that the presence of a single Cys residue within a peptide displayed on M13 pIII is an important factor in Sec insertion. The occurrence of single Cys residues in selenopeptides was over 4%, higher than both the normally observed (<0.5%) and predicted (3.1%) frequencies for similar displayed peptide libraries. Moreover, library clones containing a single Cys residue possessed opal codon

mutations with <1% frequency compared to almost 10% for the entire TGAT library. These effects presumably resulted from seleno-sulfide cross-linking, which would stabilize both the Cys and Sec residues in the M13 display system, where the coat protein pIII folds in the periplasm. Because it was possible to obtain and amplify many stable library clones containing an unpaired Sec but not a Cys, it appears that single Sec residues are somewhat more stable than unpaired Cys residues.

Among sequences that did not contain a single Cys residue, the nucleotide immediately downstream was a critical factor in determining whether Sec or Trp insertion occurred. The TGA-purine clones replicated with comparable phage yield and sequence fidelity regardless of the media Se concentration, suggesting that Sec insertion was not the major pathway. Purines in the first downstream position have previously been shown to enhance Trp-inserting opal suppression by endogenous tRNA^{Trp} (Miller and Albertini, *supra*). Notably, the TGA CTG sequence present in the Se-independent clone TGAN-10 has also been shown to strongly promote Trp insertion (Miller and Albertini, *supra*). It was recently demonstrated that a downstream SECIS element enhanced opal suppression, presumably by Trp, even in the absence of functional SelB or SelC, possibly by interfering with RF2-dependent termination or by stabilizing the message (Suppmann, et al., *EMBO J.*, 18:2284-2293 (1999)). Although the combination of the immediate downstream purine/CTG

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and the mRNA SECIS appeared to drive the Trp insertion pathway, it did not prohibit Sec insertion. Amplification of the TGA-purine clones in Se-supplemented media permitted Sec insertion, with phage reactivity comparable to that of fully Se-dependent clones.

Clones with an immediate downstream pyrimidine, except for CTG (TGAN-10), appeared to utilize primarily the Sec insertion opal suppression pathway; they required supplemental Se in order to produce functional phage, which was reactive with I-Bt. No other opal suppression pathway was implicated, since amplification in unsupplemented media resulted in either very low phage production or opal codon mutations. The occasional tendency of these clones to acquire opal mutations during Se-supplemented amplification was consistent with the recent finding that *E. coli* Sec insertion is only about 5% efficient (Suppmann, et al., *supra*). A spontaneous mutation in the opal codon would result in more efficient phage production, so that mutants would rapidly dominate the log-phase bacterial culture.

It has been shown (Poole, et al., *EMBO J.* 14:151-158 (1995)) that the nucleotide immediately downstream of the opal codon influences translational termination efficiency in *E. coli*, with an overall order of U>G>A>C. It was proposed that the recoding event of Sec insertion is favorable at the UGAC in *E. coli* *fdh* because RF2 binding is unfavorable in this context. The present data demonstrates that any nucleotide in the

downstream position is capable of directing Sec insertion, and indeed, UGAU and UGAC are equally proficient. This suggests that the pathway leading to Sec insertion is independent of any effect of the immediate downstream nucleotide on RF2 binding.

All of the factors discussed above contribute to the observed preference for immediate downstream purines in the TGAN library. The downstream purine, followed by a SECIS, permitted maximal utilization of the endogenous opal suppression pathway without preventing cotranslational Sec insertion. This dual-pathway strategy effectively maximizes phage production. The fixed downstream pyrimidine (TGAT) library clones strongly favor the Sec insertion pathway, presumably resulting in more homogeneous displayed peptides. The cost of this homogeneity, however, is the likelihood of selection for adventitious mutations. These issues should be considered in cloning strategies for the bacterial expression of Sec-containing peptides and proteins.

EXAMPLE V

A SELENOSULFIDE-CONSTRAINED PEPTIDE LIBRARY

Disulfide-constrained peptide libraries have been widely used for the discovery of high-affinity ligands for a number of targets (Giebel, et al., *Biochemistry*, 34:15430-15435 (1995); McLafferty, et al., *Gene* 128:29-36 (1993); O'Neil, et al., *Proteins* 14:509-515 (1992)). Flanking the randomized

sequence with cysteine residues results in spontaneous oxidation of the thiol groups in aqueous buffer to form a disulfide crosslink. This results in the display of each peptide in the library as a disulfide-constrained loop, improving the free energy of binding by lowering the unfavorable entropic change associated with binding a free peptide to a target. Additionally, libraries of this type have proven useful in the identification of structural epitopes for antibodies (Luzzago, et al., *Gene* 128:51-57 (1993), and mimotopes (McConnell, et al., *Gene* 151:115-118 (1994)).

A drawback of disulfide-constrained libraries is that the disulfide crosslink is not stable under mildly reducing conditions, as are required by redox-sensitive protein targets such as bacterial cytoplasmic proteins. Under conditions where these targets would be expected to be stable, e.g., 10 mM dithiothreitol (DTT), a cysteine flanked peptide library would be linear and unstructured, rather than constrained. A solution to this problem is to replace one of the cysteines with selenocysteine (Sec), resulting in a spontaneous selenosulfide (Se-S) crosslink which would be stable under mildly reducing conditions. Using the present invention as embodied in Examples I-III described herein, a Sec encoding UAG opal codon, with an appropriately spaced SECIS element, can be incorporated on one side of a randomized segment of codons. A cysteine codon (UGU or UGC) is introduced on the other side, resulting the in the randomized segment being

structurally constrained by a redox-stable selenosulfide crosslink (Figure 11).

As a demonstration of this technique a known ligand-
5 target pair was chosen in which a disulfide constraint was previously shown to enhance binding of the ligand to the target. By flanking the ligand sequence with a pair of cysteines, or cysteine and selenocysteine, it was expected that both would bind the target well under nonreducing
10 conditions, but only the selenosulfide-constrained sequence would bind well under reducing conditions. The ligand-target pair chosen was the sequence Cys-HPQGPP-Cys, (SEQ ID NO:36) which was demonstrated to bind streptavidin with 65-fold higher affinity than the linear sequence Ser-HPQGPP-Ser
15 (SEQ ID NO:36) (Giebel, et al., *supra*).

The following oligonucleotides were synthesized, purified, annealed, extended and ligated into M13KE as described in Example I (Eag I and Acc65 I sites underlined):

20 Ser-Ser: 5'-CATGTTTCGGCCGATTGATGAAGCCCAGCCACGCT
TGGGCCGTGGCTCGGTGGACCTTGCGGATGGCTTCCGCAGAGTG
AGAATAGAAAGGTACCCGGG-3' (SEQ ID NO:37)

Cys-Cys: 5'-CATGTTTCGGCCGATTGATGAAGCCCAGCCACGCTTG
25 GGCCGTGGCACGGTGGACCTTGCGGATGGCATTCCGCAGAGTGAGA
ATAGAAAGGTACCCGGG-3' (SEQ ID NO:38)

Cys-Sec: 5'-CATGTTTCGGCCGATTGGTGCAGACCTGCAACCGAT
GGGCCGTGTCACGGTGGACCTTGCGGATGGCATTCCGCAGAGTGA
GAATAGAAAGGTACCCGGG-3' (SEQ ID NO:39)

5 All three inserts encode the same sequence HPQGPP
(SEQ ID NO:36), but flanked by Ser-Ser, Cys-Cys and Cys-Sec
as indicated. The Cys-Sec insert has the *E. coli fdh* SECIS
immediately downstream of the UGA opal codon, while the
other inserts have the same amino acid sequence encoded by
10 the SECIS but a different nucleotide sequence, abolishing any
selenocysteine-directing activity. To enhance selenocysteine
incorporation, media contained 2 μ M sodium selenite in all
plating and amplification steps for the Cys-Sec construct, but
not the others.

15 Following electroporation into *E. coli* ER2738, plaques
were picked and amplified in 20 ml early-log cultures of
ER2738 for 5 hours at 37°C. Cells were removed by
centrifugation and phage recovered from the supernatant by
20 overnight precipitation with 1/6 volume 20% polyethylene
glycol 8000 in 2.5 M NaCl at 4°C. Following centrifugation and
reprecipitation, phage were suspended in 100 μ l Tris-buffered
saline (TBS), pH 8 and titered for plaque forming units. DNA
sequencing indicated that the phage were displaying the
25 correct sequences, with the exception of the Cys-Sec phage,
which carried a point mutation which resulted in the displayed
sequence being Cys-HPQGPT-Sec (SEQ ID NO:42), rather than
Cys-HPQGPP-Sec.

Polystyrene plates were coated overnight with streptavidin (Prozyme, San Leandro, CA) at a concentration of 100 µg/ml in 0.1 M bicarbonate buffer, pH 8.6. Plates were blocked with 1 mg/ml bovine serum albumen in TBS and washed with TBS containing 0.05% Tween-20 (TBST). Phage were diluted in TBS either containing 10 mM dithiothreitol (DTT) or not containing DTT, and applied to the blocked, streptavidin-coated wells. After a 2 h incubation at 20°C, plates were washed extensively with TBST and bound phage were detected with anti-M13 antibody conjugated to horseradish peroxidase (Amersham-Pharmacia, Piscataway, NJ), following the instructions provided by the manufacturer.

The results (Figure 12) clearly show that binding of the Cys-Cys sequence to streptavidin is reduced by at least two orders of magnitude in the presence of 10 mM DTT. In contrast, both the linear (Ser-Ser) and the selenosulfide-constrained (Cys-Sec) sequences bind more tightly in the presence of DTT, possibly due to partial unfolding of pIII, which has 4 disulfide bonds, which may increase target accessibility of the displayed peptide. Importantly, the Cys-Sec sequence binds 2 orders of magnitude more tightly than the linear Ser-Ser sequence both in the presence and the absence of DTT, indicating the presence of the selenosulfide

crosslink. The reduced binding of the Cys-Sec sequence compared to the Cys-Cys sequence in the absence of DTT is likely due to the point mutation which altered a proline in the reported sequence (Giebel, et al., *supra*) to a threonine.

5 Taken together, these data demonstrate that a selenosulfide-constrained peptide is stable under conditions (10 mM DTT) where the corresponding disulfide-constrained sequence is reduced to the poorly-binding linear form. It can therefore be inferred that the selenosulfide crosslink would impart the
10 same redox stability to a constrained peptide library as the sequences described here, allowing discovery of constrained peptide ligands even under reducing conditions.

WHAT IS CLAIMED IS:

1. A peptide comprising selenocysteine, wherein the peptide is fused to a surface protein of an amplifiable genetic particle.
2. The peptide of claim 1, wherein the amplifiable genetic particle is selected from the group consisting of phage, polysomes, virus, cells and spore.
3. The peptide of claim 1, wherein the peptide is fused to the surface protein.
4. A surface protein of an amplifiable genetic particle into which has been incorporated a selenocysteine residue.
5. The peptide of claim 4, wherein the amplifiable genetic particle is selected from the group consisting of phage, polysomes, virus, cells and spore.
6. A method for incorporating a selenocysteine residue on the surface of an amplifiable genetic particle comprising the steps of:
 - (a) incorporating a codon selected from the group consisting of TGA and UGA into a predetermined position of a nucleic acid molecule which codes for a peptide located on the surface of an amplifiable genetic particle; and

(b) incorporating a selenocysteine insertion sequence at a predetermined position downstream from the codon to form a selenocysteine expression cassette.

- 5 7. The method of claim 6, wherein the selenocysteine insertion sequence is obtainable from the group consisting of eubacteria, eukarya and archaea.
- 10 8. The method of claim 6, wherein the nucleic acid molecule comprising the selenocysteine expression cassette is genetically fused to a nucleic acid molecule coding for a surface peptide of an amplifiable genetic particle.
- 15 9. The method of claim 6, wherein the nucleic acid molecule comprising the selenocysteine expression cassette is
- (a) expressed to produce a selenopeptide; and
- (b) ligated to the surface of an amplifiable genetic particle.
- 20 10. A method of modifying a selenocysteine containing peptide on the surface of an amplifiable genetic particle comprising chemical derivitization of a selenol group of the selenocysteine containing peptide.
- 25 11. The method of claim 10, wherein chemical derivitization comprises a nucleophilic substitution reaction.

12. The method of claim 10, wherein chemical derivitization comprises an oxidation reaction.

13. The method of claim 10, wherein chemical derivitization
5 comprises a metal coordination reaction.

14. The method of claim 10, wherein chemical derivitization
comprises introduction of a chemical functionality selected
from the group consisting of enzyme substrates, enzyme
10 cofactors, enzyme inhibitors, and cytotoxic agents.

15. A randomized peptide library comprising a fixed
selenocysteine residue flanked on at least one side by a
randomized amino acid on the surface of an amplifiable
15 genetic particle.

16. A method of selecting for novel ligands comprising the
steps of:

(a) chemical derivitization of a selenocysteine residue
20 in a random peptide library displayed on the surface of an
unamplifiable genetic particle to form a chemically modified
peptide library;

(b) reacting the chemically modified peptide library
with a target molecule;

(c) removing unbound particles;

(d) eluting bound particles; and

(e) identifying peptide sequence displayed on the
25 eluted bound particles.

17. A method for selecting for a predetermined enzyme activity comprising the steps of:

- 5 (a) displaying a selenopeptide on the surface of an amplifiable genetic particle;
- (b) displaying a library of enzyme variants on the amplifiable genetic particle of step (a);
- (c) chemically derivitizing the selenopeptide from step (a) with a predetermined substrate;
- 10 (d) reacting the chemically derivitized particles from step (c) with an affinity matrix specific for a product corresponding to the predetermined enzyme activity other than the substrate of the enzyme;
- (e) removing unbound particles;
- 15 (f) eluting bound particles; and
- (g) identifying enzymes displayed on the eluted bound particles.

18. A method of identifying required DNA sequence elements for incorporation of selenocysteine into peptides comprising the steps of:

- 20 (a) fusing a selenocysteine expression cassette to a surface peptide of an amplifiable genetic particle, whereby expression of the surface peptide is dependent upon incorporating a selenocysteine residue;
- 25 (b) forming a library of sequence variants of the selenocysteine expression cassette; and

(c) selection for particles which are genetically amplifiable.

5 19. A structurally constrained peptide library displayed on the surface of an amplifiable genetic particle comprising one or more randomized amino acid residues flanked by a cysteine residue on one side and a selenocysteine residue on the other side, said constraint resulting from a spontaneously formed selenosulfide cross-link.

10 20. A method for discovery of structurally constrained ligands for a target molecule comprising the following steps:

(a) reacting a structurally constrained peptide library displayed on the surface of an amplifiable genetic particle,
15 comprising one or more randomized amino acid residues flanked by a cysteine residue on one side and a selenocysteine residue on the other side, with a target molecule;

(b) removing unbound particles;

20 (c) eluting bound particles; and

(d) identifying peptide sequence displayed on the eluted bound particles.

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(57) Abstract

The naturally-occurring amino acid selenocysteine (SEC) is incorporated uniquely and specifically in the context of a polypeptide displayed on the surface of an amplifiable genetic particle (phage, cell or spore) in response to incorporation signals engineered in the encoding DNA. In addition to conferring the unique activities of the selenol group to the chemistry of the displayed peptide, Sec also provides a unique handle for specific chemical modification of the display peptide. In addition to increasing the palette of available residues in a random peptide library to 21 possibilities, present invention also provides a means of tethering virtually any desired chemical functionality to the incorporated Sec.

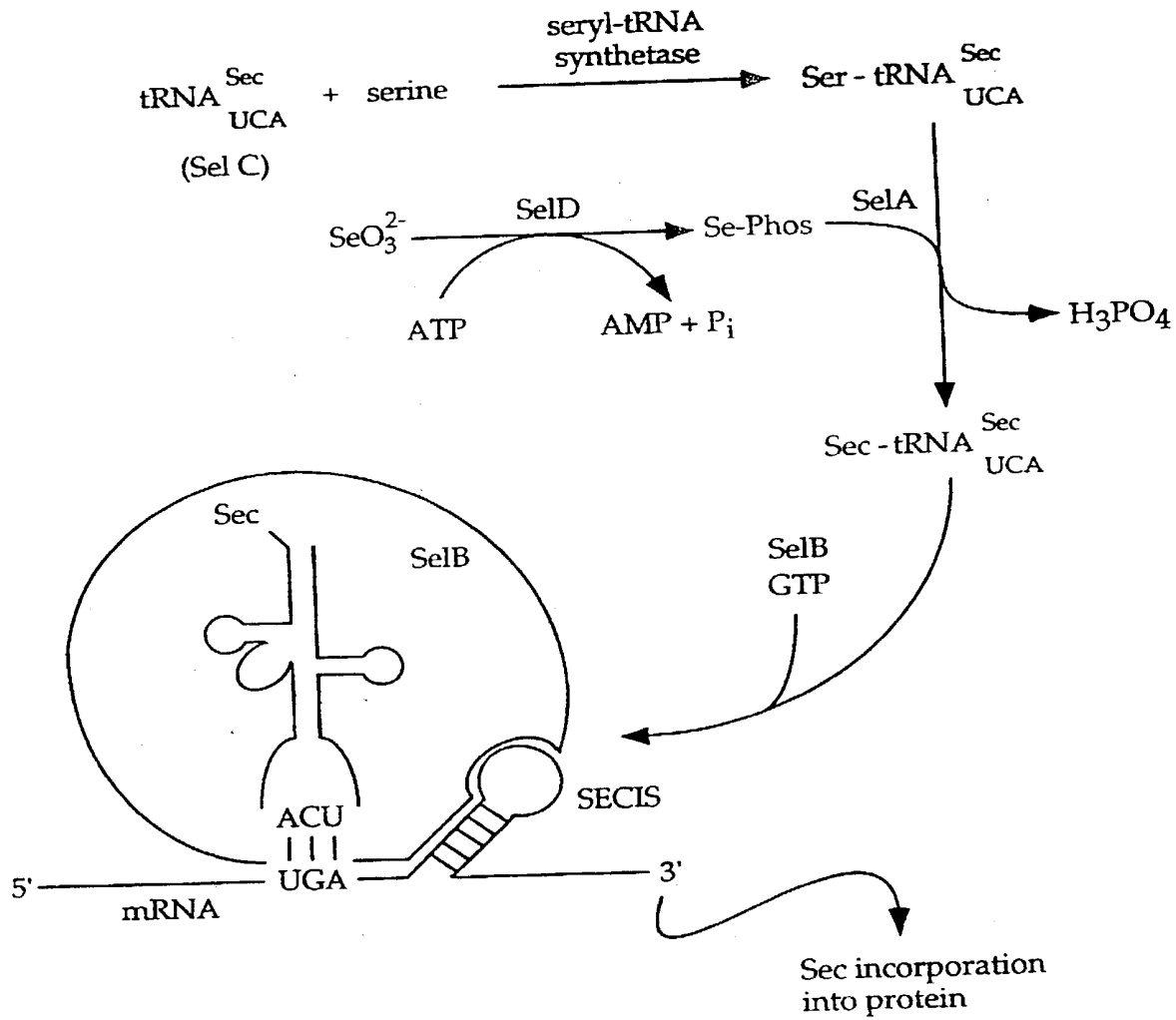


Figure 1

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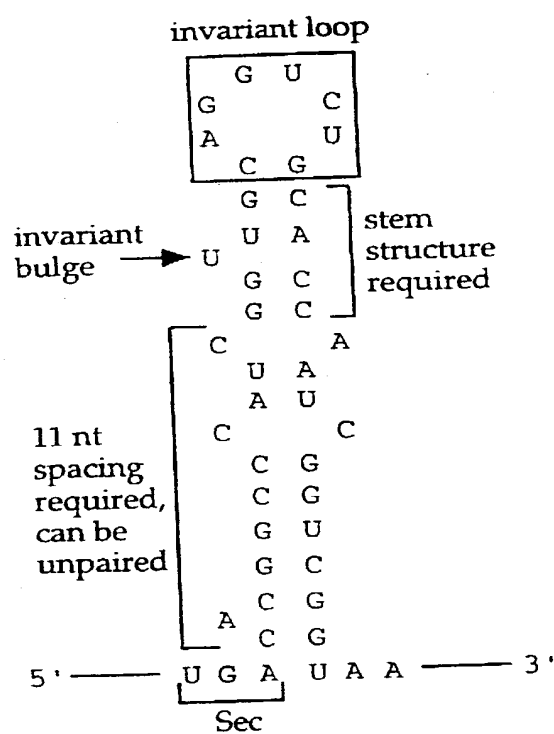
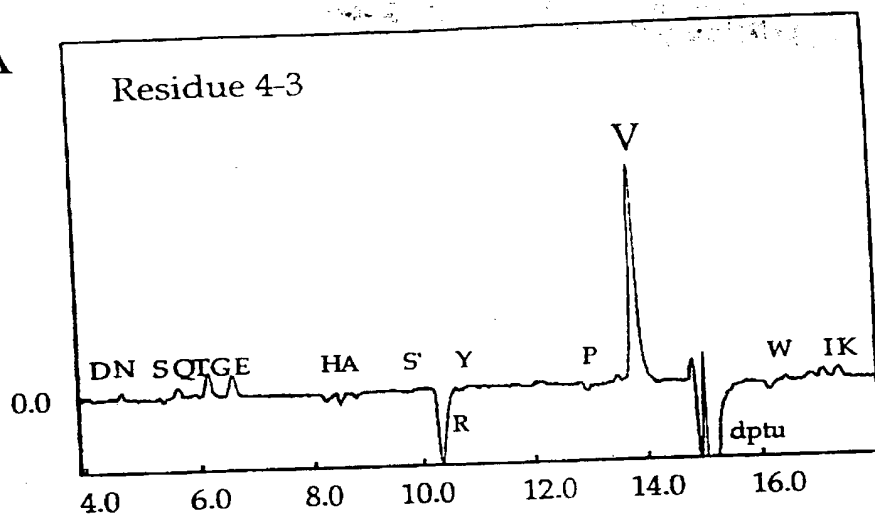


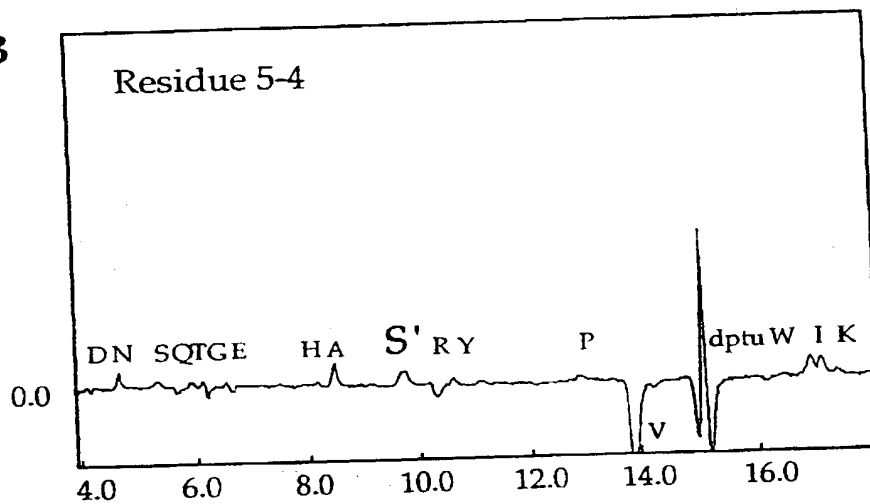
Figure 2

09/937187

A



B



C

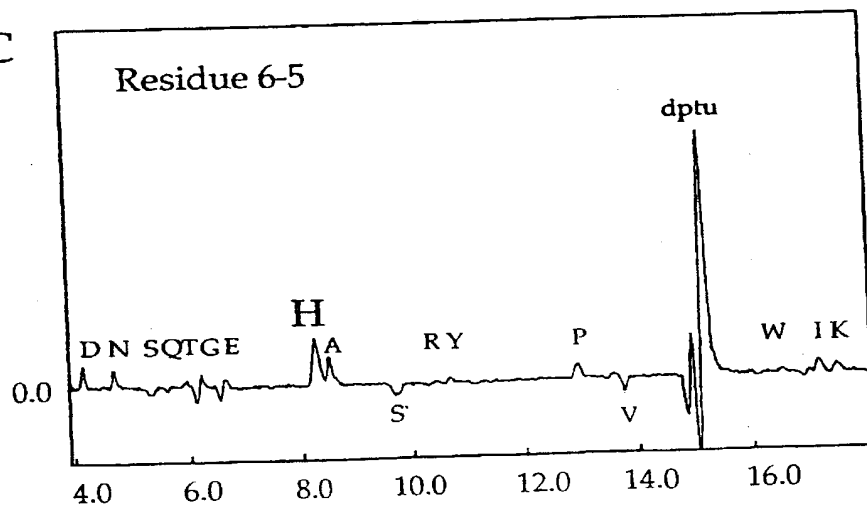


Figure 3

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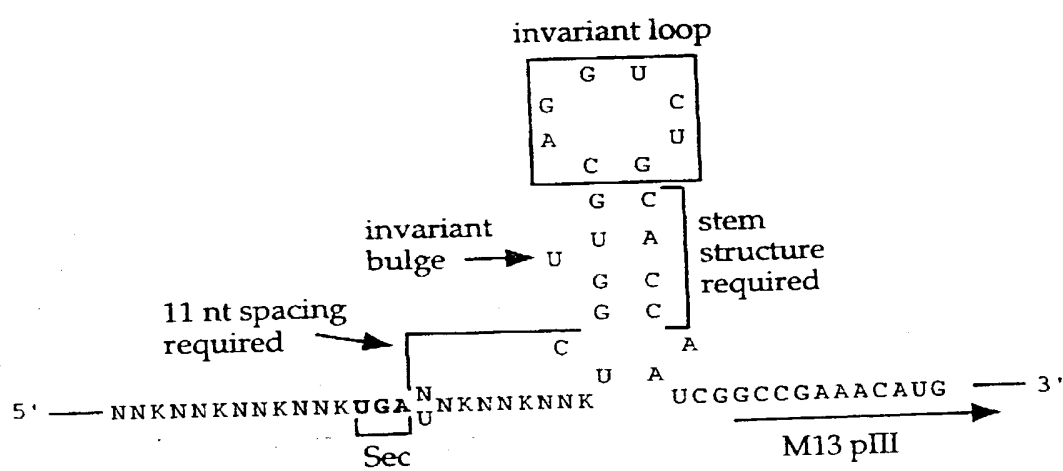


Figure 4

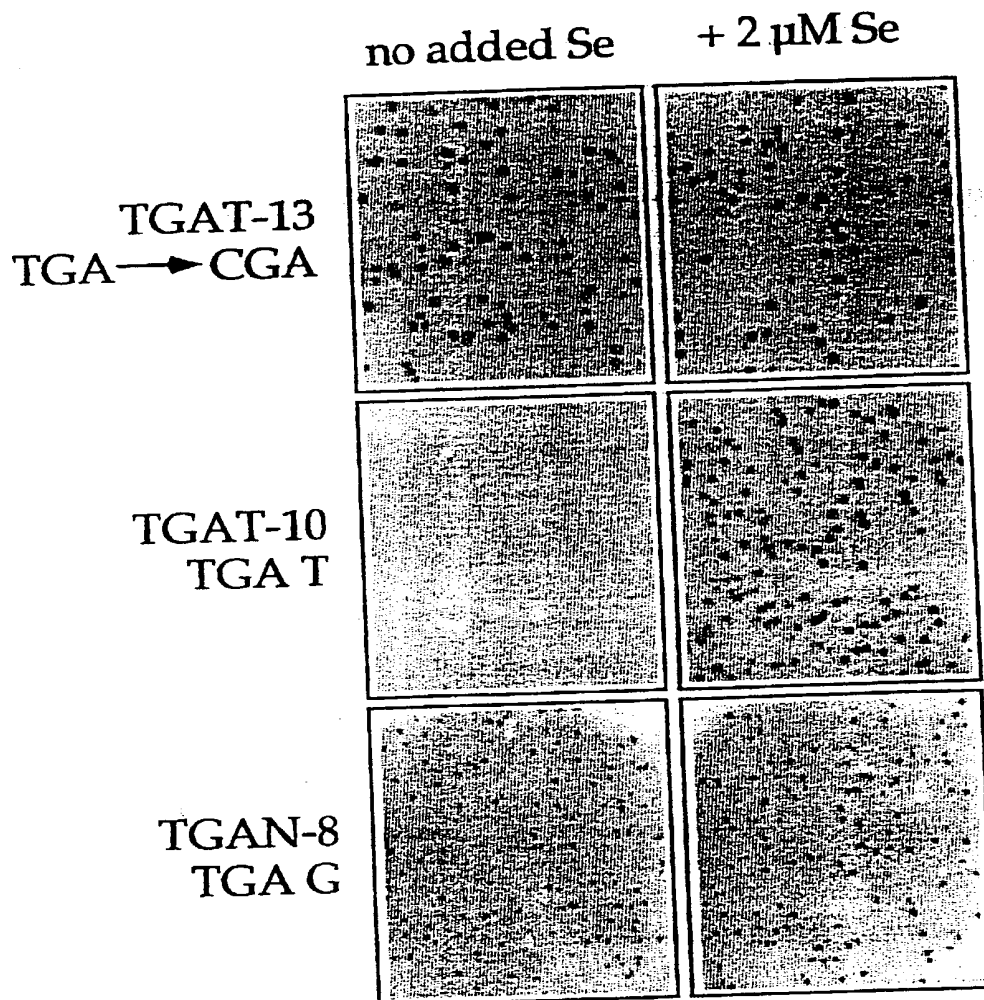


Figure 5

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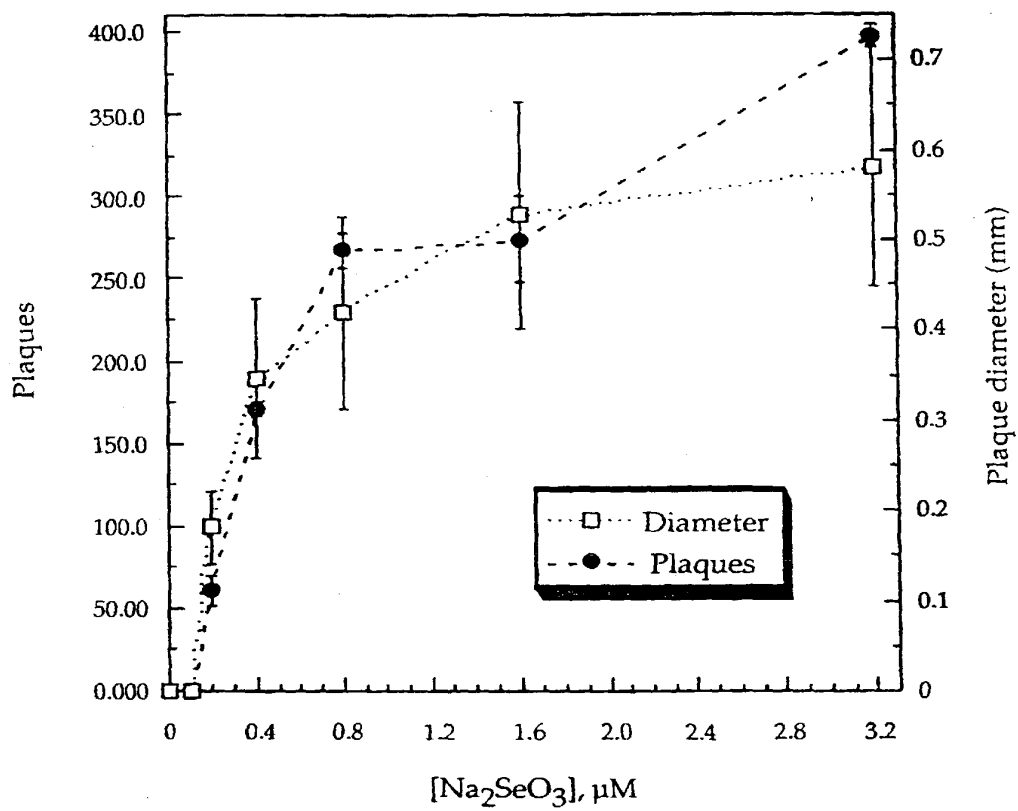


Figure 6

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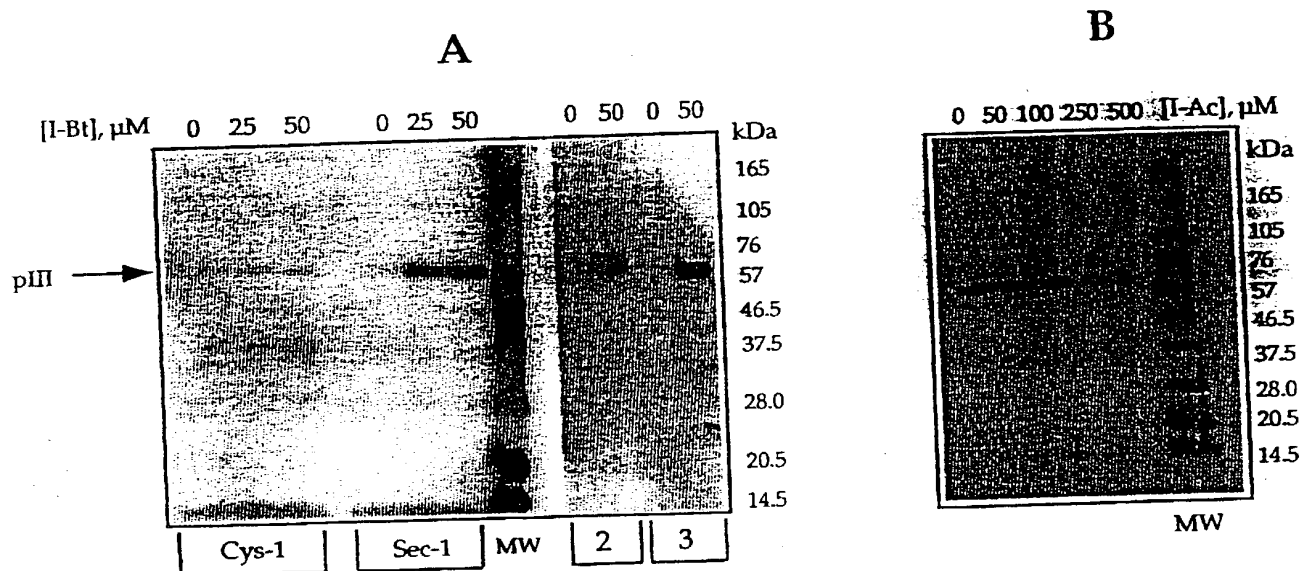
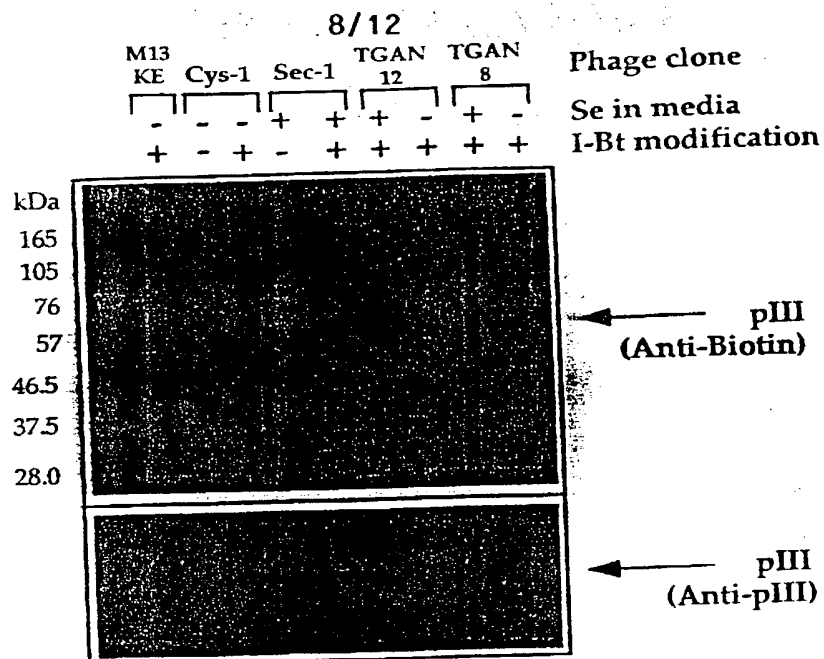


Figure 7

A



B

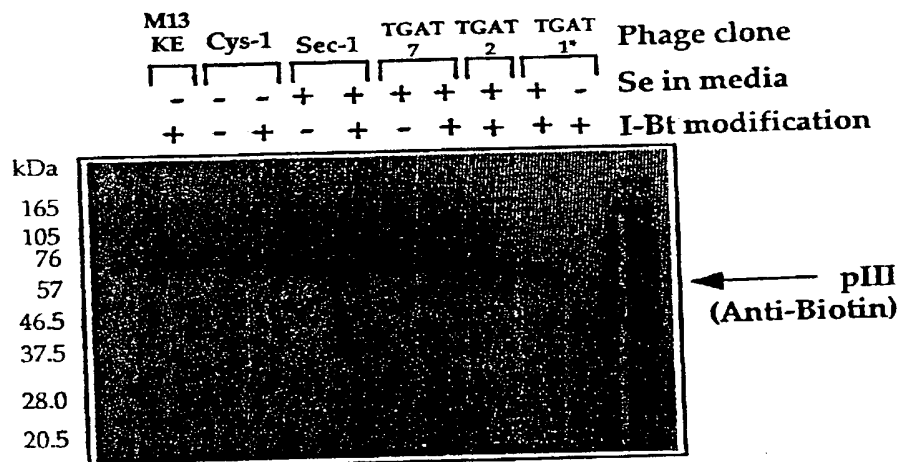


Figure 8

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Fig 9

| Clone | Random insert | | | | | | | | Se- dependent? |
|---------|---------------|-----|-----|-----|-----------------|-----|-----|-----|-------------------|
| TGAN-1 | TCG | TCT | TTT | CCT | TGA | AAG | TCG | CCT | - |
| | S | S | F | P | op ^a | K | S | P | |
| TGAN-2 | AAG | TGT | ACG | CTT | TGA | TCT | ATG | CTG | + |
| | K | C | T | L | Sec | S | M | L | |
| TGAN-3 | TTG | CTT | TTG | CCT | TGA | AAT | GTT | CTT | - |
| | L | L | L | P | op | N | V | L | |
| TGAN-4 | ATG | ACT | ACG | CAG | TGA | CCT | TCT | CTG | + |
| | M | T | T | Q | Sec | S | M | L | |
| TGAN-5 | CAT | ATT | CCG | CCG | TGA | ACG | AAT | CCT | - |
| | H | I | P | P | op | T | N | P | |
| TGAN-6 | AAG | GCT | CTG | TGT | TGA | CAG | GAT | TCG | + |
| | K | A | L | C | Sec | Q | D | S | |
| TGAN-7 | CTT | CTT | CCG | TGT | TGA | GCT | CAG | CCG | + ^b |
| | L | L | P | C | Sec | A | Q | P | |
| TGAN-8 | CAT | CAT | CCG | ACT | TGA | GCT | AAG | CAG | - |
| | H | H | P | T | op | A | K | Q | |
| TGAN-9 | ATG | CCT | CCT | ACG | TGA | ATG | GCT | ACG | - |
| | M | P | P | T | op | M | A | T | |
| TGAN-10 | AAT | TGG | TTT | TCT | TGA | CTG | ACT | ACG | - |
| | N | W | F | S | op | L | T | T | |
| TGAN-11 | CTG | CAT | CCG | ACG | TGA | GCT | CGG | CCT | - |
| | L | H | P | T | op | A | R | P | |
| TGAN-12 | GAT | AGG | GGG | CCT | TGA | GCG | AAG | ATT | - |
| | D | R | G | P | op | A | K | I | |
| TGAN-13 | GCG | TCT | TTG | CCT | TGA | AGG | ACG | AGT | - |
| | A | S | L | P | op | R | T | S | |

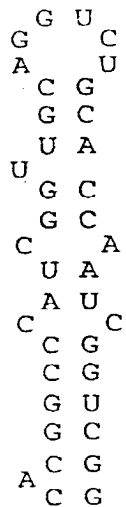
^aop: opal suppression: Sec or W, depending on Se availability^bTGAN-7 production was Se-enhanced; Se supplementation yielded larger (3-4x diameter) and more (10x) plaques.~~Table 1~~ Selected Library TGAN clones. TGAN-1 was expressed as a pMal-pIII fusion, and tryptophan incorporation was verified by N-terminal sequencing.

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Fig 10

| Clone | Random insert | | | | | | | | Se- dependent? |
|---------|---------------|-----|-----|-----|-------|-----|-----|-----|-------------------|
| TGAT-1 | L | P | R | Q | Sec W | S | P | | + |
| | TTG | CCG | CGT | CAG | TGA | TGG | TCT | CCG | |
| TGAT-2 | L | T | G | T | Sec C | Q | N | | + |
| | TTG | ACT | GGT | ACG | TGA | TGT | CAG | AAT | |
| TGAT-3 | E | A | S | R | Sec C | S | T | | + |
| | GAG | GCG | TCG | CGT | TGA | TGT | TCG | ACT | |
| TGAT-4 | K | L | A | R | Sec S | A | S | | + |
| | AAG | TTG | GCT | CGT | TGA | TCG | GCG | TCG | |
| TGAT-5 | N | G | A | Q | Sec S | R | H | | + |
| | AAT | GGG | GCG | CAG | TGA | TCG | AGG | CAT | |
| TGAT-6 | A | S | P | T | Sec F | K | P | | + |
| | GCG | AGT | CCT | ACT | TGA | TTT | AAG | CCG | |
| TGAT-7 | C | A | H | P | Sec S | T | R | | + |
| | TGT | GCT | CAT | CCG | TGA | TCT | ACT | CGT | |
| TGAT-8 | Q | S | T | R | Sec W | N | D | | + |
| | CAG | TCG | ACG | CGG | TGA | TGG | AAT | GAT | |
| TGAT-9 | I | V | E | S | Sec L | N | P | | + |
| | ATT | GTG | GAG | TCG | TGA | TTG | AAT | CCG | |
| TGAT-10 | T | Q | R | M | Sec L | P | P | | + |
| | ACG | CAG | CGT | ATG | TGA | TTG | CCG | CCC | |
| TGAT-11 | V | Q | Y | T | Sec L | P | K | | + |
| | GTG | CAG | TAT | ACG | TGA | TTG | CCG | AAG | |
| TGAT-12 | A | G | Q | S | Sec S | T | D | | + |
| | GCT | GGG | CAG | TCG | TGA | TCG | ACT | GAT | |
| TGAT-13 | L | S | A | S | R | S | Q | F | - |
| | CTG | TCT | GCG | AGT | CGA | TCG | CAG | TTT | |

~~Table 2.~~ Selected TGAT library clones. TGAT-13 carried a T → C point mutation within the opal codon.



5' - GCGUGCNNKNNKNNKNNKNNKNNKNNKUGA, UAA — gene III

Sec

Translation/
Spontaneous oxidation



Figure 11

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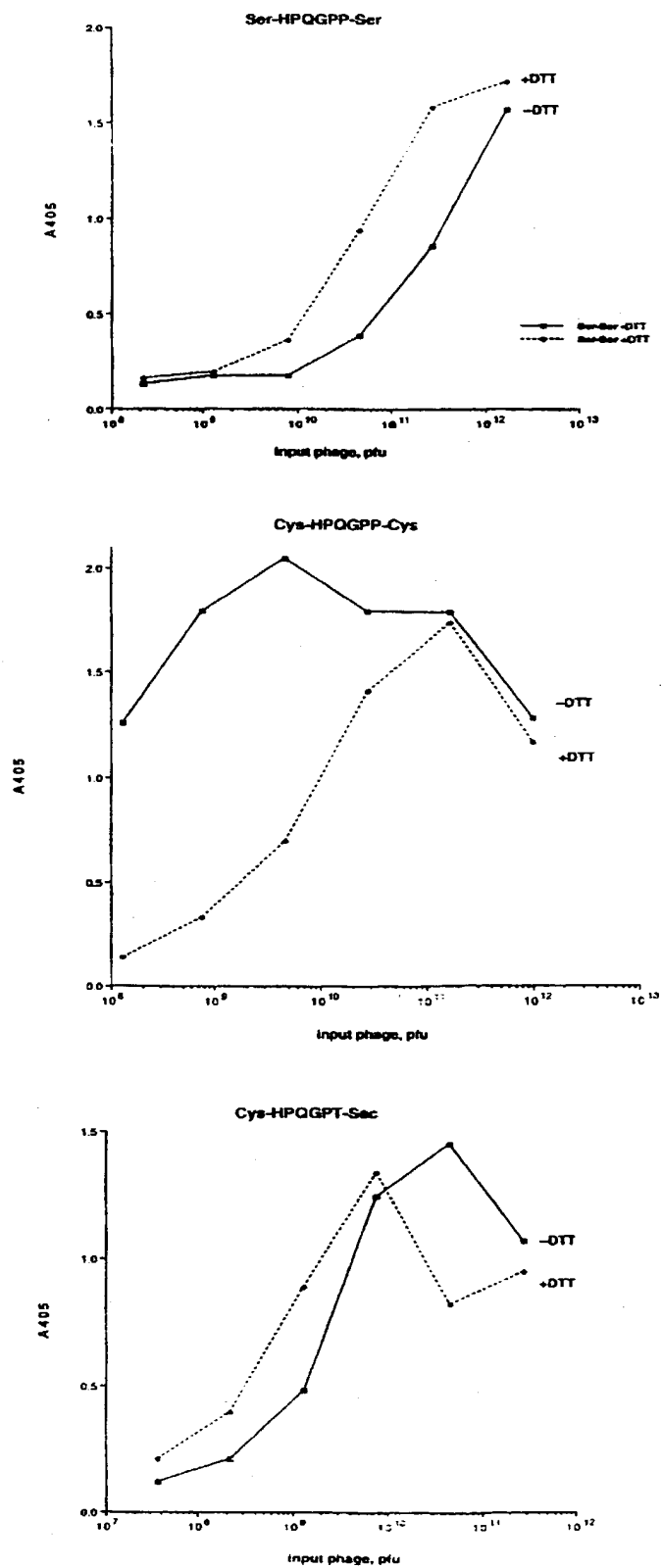


FIGURE 12

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**DECLARATION
AND POWER OF ATTORNEY
Original Application**

Attorney Docket No. NEB-164-PUS

As a below named inventor, I hereby declare that:

My residence, post address and citizenship are as stated below next to my name

I believe that I am the original, first and sole inventor (in only one name is listed at 201 below) or an original, first and joint inventor (if plural names are listed at 201-203 below) of the subject matter which is claimed and which a patent is sought on the invention entitled:

Surface Display of Selenocysteine-Containing Peptides

which is described and claimed in:

☒ the attached specification or ☐ the specification in Application Serial No. _____ filed _____
(for declaration not accompanying application)
And was amended on _____
if applicable

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendments referred to above. I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a). I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

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|---|----------------|--------------------------------------|---|
| COUNTRY | APPLICATION | DATE OF FILING (day, month, year) | PRIORITY CLAIMED UNDER 35 U.S.C. 119 |
| | | | YES NO |
| | | | YES NO |
| ALL FOREIGN APPLICATION(S) IF ANY, FILED MORE THAN 12 MONTHS PRIOR TO THE FILING DATE OF THIS APPLICATION | | | |
| COUNTRY | APPLICATION | (day, month, year) | PRIORITY CLAIMED UNDER 35 U.S.C. 119 |
| PCT | PCT/US00/13292 | 12 May 2000 | |
| | | | |

I hereby claim the benefit under Title 35, United States Code §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

| Application Serial No. | Filing Date | Status (Patented, Pending, Abandoned) |
|------------------------|-------------|---------------------------------------|
| 60/134,286 | 14 May 1999 | |
| | | |
| | | |
| | | |
| | | |

DECLARATION
AND POWER OF ATTORNEY
PAGE 2 OF 3

POWER OF ATTORNEY:

As a named inventor, I hereby appoint the following attorney with full powers of association, substitution and revocation to prosecute this application and transact all business in the Patent and Trademark Office connected therewith:

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PAGE 3 OF 3

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| 209 | Full Name of Inventor | Last Name | First Name | Middle Name |
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I hereby further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true and further that these statements were made with the knowledge that willful statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

| | |
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| Signature of Inventor 202 <i>Chen H. Hoven</i> | Date 9/20/01 |
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| Signature of Inventor 204 | Date |
| Signature of Inventor 205 | Date |
| Signature of Inventor 206 | Date |
| Signature of Inventor 207 | Date |
| Signature of Inventor 208 | Date |
| Signature of Inventor 209 | Date |

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NEW ENGLAND BIOLABS, INC.

<120> SURFACE DISPLAY OF SELENOCYSTEINE-CONTAINING PEPTIDES

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<150> 60/134,286

<151> 1999-05-14

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<170> PatentIn Ver. 2.0

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WO 00/70100

PCT/US00/13292

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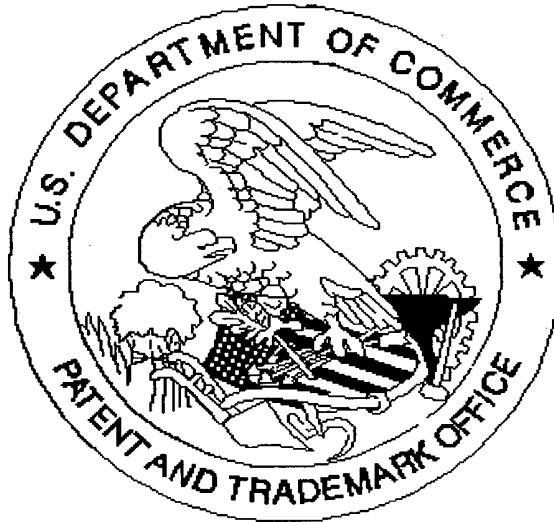
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Office of Initial Patent Examination -- Scanning Division



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for scanning. (Document title)

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